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Antigenic sites of the nicotinic acetylcholine receptor cannot be predicted from the hydrophilicity profile

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The amino acid sequences of the polypeptide chains of the acetylcholine receptor have recently been published. From the hydrophilicity profiles, it has been proposed that residues 161–166 of the α-chain might be an important antigenic site. We have synthesised a peptide containing this sequence and raised antisera to it. Here we report that this peptide does not represent an important antigenic site on the molecule, and that this region is probably inaccessible to antibodies. Based on the known DNA sequences and hydrophilicity profiles of the receptor chains, we suggest that many regions of high hydrophilicity may represent inter-domain regions of proteins.

Antigenic receptor *Acetylcholine* *Hydrophilicity* *Intron* *Peptide*

1. INTRODUCTION

The nicotinic acetylcholine receptor (nAChR) has been the subject of intensive research in recent years, not only for its role as a receptor but also as the major autoantigen in the human disease, myasthenia gravis [1]. The receptor can be readily purified to homogeneity from the electric organs of fish, such as the ray, *Torpedo*. The purified material consists of 4 different polypeptide chains ($\alpha, \beta, \gamma, \delta$) held together by noncovalent forces [2]. Working with a battery of monoclonal antibodies to the nAChR of *Torpedo californica*, authors in [3,4] have demonstrated that most antibodies to the nAChR are directed to a compact region which they call the main immunogenic region (MIR), and that these antibodies bind to a certain extent to the α-chain. Recently, the genes for all the chains of *T. californica* and the α-chain of calf and human nAChR have been cloned and sequenced [5–9]. A very similar sequence has been obtained for the α-chain of the nAChR from another ray, *T. marmorata* [10,11]. Extreme conservation of the primary structure of the chain is found across these different species. Based on the hydrophilicity profile of the amino acid sequence of the α-chain of *T. californica*, it has been proposed that a region consisting of residues 161–166 might correspond to the MIR [5,8,9]. We have synthesised a peptide containing the sequence 151–169 of the chain of *Torpedo* and here report that this site does not represent an important antigenic site on the nAChR.

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Abbreviations: Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; DCCD, dicyclohexylcarbodiimide; DMF, dimethylformamide; Hobt, 1-hydroxybenzotriazole; TEA, triethylamine; nAChR, nicotinic acetylcholine receptor; BSA, bovine serum albumin; RSA, rabbit serum albumin; KLH, keyhole limpet haemocyanin; NRS, normal rabbit serum; MIR, main immunogenic region; PBS, phosphate-buffered saline (pH 7.1); PBE, PBS containing 0.1% (w/v) BSA and 1% (v/v) Emulphogen (pH 7.1)

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2. MATERIALS AND METHODS

2.1. *Synthesis and characterisation of peptide 151–169 (Tyr-Asp-Gly-Thr-Lys-Val-Ser-Ile-Ser-Pro-Glu-Ser-Asp-Arg-Pro-Asp-Leu-Ser-Thr)*

Boc–L-amino acid derivatives were purchased from either Senn Chemicals or Fluka. Side chain protecting groups were ϵ-benzyloxycarbonyl group for lysine, γ and β-benzyl esters for glutamic and aspartic acid, respectively, *o*-benzyl for serine and threonine and finally nitro group for arginine. The purity of these derivatives was checked by thin-layer chromatography. The standard procedure of solid-phase peptide synthesis [12] was modified as follows: Firstly, mixing was achieved by flushing nitrogen through the reaction vessel; secondly, alternate washing with solvents which favour swelling (methylene chloride) and extreme shrinking (methanol) has been favoured [13].

The C-terminal residue, Thr(OBzl), was esterified to chloromethylated polystyrene (1% divinylbenzene) [14] and the substitution was found to be 0.30 mmol/g resin on the basis of free amino group (upon deblocking of the Boc group) determined using picric acid [15].

The protocol for a typical amino acid coupling includes the use of 10–15 ml solvent/g resin (only 3–5 ml/g for the coupling step) and mixing (N_2 flushing) for 2 min, unless otherwise specified. (a) 50% trifluoroacetic acid/CH_2Cl_2; (b) 50% trifluoroacetic acid/CH_2Cl_2, 30 min; (c) CH_2Cl_2 3×; (d) $CHCl_3$ 3×; (e) TEA/$CHCl_3$ (1:9), 5 min; (f) TEA/$CHCl_3$ (1:9), 20 min; (g) $CHCl_3$ 3×; (h) CH_2Cl_2 3×; (i) DMF 2×; (j) 8 equiv. Boc amino acid and Hobt in DMF; (k) 8 equiv. DCCD in CH_2Cl_2, 120 min; (l) DMF 3×; (m) CH_2Cl_2 2×; (n) CH_3OH 3×; (o) CH_2Cl_2 3×; (p) second coupling of the same amino acid, steps j–o; (q) 1% *N*-acetylimidazole and 10% acetic anhydride in CH_2Cl_2, 30 min; (r) CH_2Cl_2 4×. Free NH_2 groups were measured using ninhydrin after each amino acid coupling step [16].

2.2. *Hydrogen fluoride cleavage and deprotection*

Protected peptide resin (840 mg) was treated with 1 ml anisol and about 10 ml anhydrous hydrogen fluoride for 1 h at 0°C. Hydrogen fluoride was then removed under reduced pressure. The resin was washed 3 times with ethyl acetate and the peptide extracted from the resin with 3 portions (15 ml) of 50% acetic acid and lyophilised.

2.3. *Purification*

The crude peptide was dissolved in 0.02 M ammonium acetate (pH 6.5) containing 7 M urea and applied to a column (0.7 × 26 cm) of DEAE–Sephadex A-25 equilibrated with the same buffer. A linear gradient elution was performed using 200 ml each of 0.02 M and 0.5 M ammonium acetate (pH 6.5), containing 7 M urea. One major peak appeared in the elution profile and was pooled as indicated (fig.1), desalted on Sephadex G-10 and lyophilised.

2.4. *Characterisation*

Amino acid analysis was performed after hydrolysis in constant-boiling-point HCl at 110°C for 24 h [17]. Descending paper chromatography was performed on Whatman 3M paper in butanol–acetic acid–water (4:1:5) (organic phase only). High-pressure reverse-phase liquid chromatography was carried out using a gradient of acetonitrile (0–60% in 80 min) in 0.05% trifluoroacetic acid/0.025% TEA, the flow rate being 1.0 ml/min.

2.5. *Purification and treatment of nAChR*

The nAChR from *T. marmorata* was purified as in [18] to a specific activity of 6000–10000 pmol toxin binding sites/mg protein. Receptor preparations were assayed using binding of iodinated α-bungarotoxin [19]. Acid denaturation was performed by dialysing the receptor for 1 h at 4°C against 100 vols of 1 M acetic acid, followed by dialysis against PBS.

2.6. *Iodination procedures*

Iodination of the peptide to a specific radioactivity of 400 Ci/mmol was performed using chloramine T. All reagents were in 50 mM potassium phosphate buffer (pH 7.0); 10 μl of peptide (5 μg), 10 μl $Na^{125}I$ (1 mCi) and 10 μl chloramine T trihydrate (50 μg) were mixed for 1 min at 22°C. Two hundred μl sodium metabisulphite (120 μg), 200 μl KI (2 mg) and 500 μl buffer containing 1% (w/v) BSA were added, and the sample fractionated on a column (0.9 × 25) of Sephadex G-15 in buffer and 1% BSA.

One-ml fractions were collected and the peptide peak pooled and stored at 4°C. Iodination of the nAChR to a specific radioactivity of 3500 Ci/mmol with full retention of antigenicity was performed as in [20]. Acid-denatured nAChR was iodinated by the same method. α-Bungarotoxin was labelled to a specific radioactivity of 900 Ci/mmol [21].

2.7. *Coupling of peptide to carrier protein, and immunisation protocols*

Peptide was coupled by glutaraldehyde treatment to the carrier proteins KLH and RSA using 6 mg peptide and 20 mg protein [22]. Free peptide was removed by dialysis. Incorporation of peptide was measured using trace-labelled peptide. The final conjugates contained 13–15% (w/w) peptide. Female New Zealand White rabbits were immunised intramuscularly with 10–1000 μg of conjugates at 3 weekly intervals, initially in Freund's complete adjuvant then in incomplete adjuvant. Antibodies to the nAChR and acid-denatured receptor were raised in rabbits as in [23]. Rat monoclonal antibodies 6, 12 and 16 were prepared and characterised as in [3].

2.8. *Antibody assays*

All assays were performed in PBE.

2.8.1. Iodinated peptide and anti-nAChR sera

One hundred μl of a 100-fold dilution of test sera or NRS was incubated with 50 μl labelled peptide (0.86 pmol) for 1 h at 22°C. The complexed material was precipitated by incubation with 50 μl of 10% (w/v) *Staphylococcus aureus*, Cowan strain I, in PBE.

2.8.2. Unlabelled peptide in competition with iodinated nAChR

Fifty μl of labelled antigen (nAChR or acid-denatured nAChR, 0.1 pmol) was mixed with 50 μl unlabelled peptide (12 μmol) or buffer, followed by 100 μl of test sera or NRS (dilutions of test sera were in 100-fold diluted NRS, the dilution used being sufficient to bind one-tenth of the offered labelled antigen in the absence of peptide). After 1 h at 22°C the bound counts were precipitated with *S. aureus* as above.

2.8.3. Antibodies produced by immunisation with peptide conjugate

One hundred μl of diluted test sera or NRS was incubated with 50 μl labelled peptide (0.86 pmol), nAChR (0.1 pmol), acid-denatured receptor (0.07 pmol) or SDS-denatured receptor (0.1 pmol) for 1 h at 22°C. The bound counts were then precipitated using *S. aureus*. When receptor labelled with iodinated toxin was used, excess iodinated toxin was incubated with the iodinated receptor for 1 h at 22°C, prior to use.

3. RESULTS

The peptide 151–169 from the sequence of the α-chain of *Torpedo* was synthesised by the solid-phase method. The final product showed a single spot on descending paper chromatography, and essentially a single peak on HPLC (fig.1). The amino acid composition was that expected for the required sequence: Asp 3.2(3), Ser 4.2(4), Thr 1.9(2), Glu 1.1(1), Pro 1.9(2), Gly 1.0(1), Val 1.0(1), Ile 1.0(1), Leu 1.0(1), Tyr 0.9(1), Lys 1.0(1), Arg 1.0(1). Binding experiments using iodinated peptide and antibodies to native and denatured receptor (table 1) demonstrated that the peptide is recognised only by antibodies to

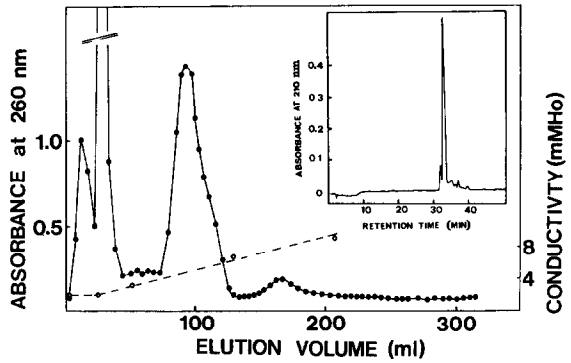

Fig.1. Ion-exchange chromatography of synthetic peptide 151–169 on a DEAE–Sephadex A-25 column (0.7 × 26 cm). See section 2 for experimental conditions. Absorbance was monitored at 260 nm. The fractions comprising the major peak were pooled as indicated, desalted on a column (2 × 40 cm) of Sephadex G-10 in 0.1 M acetic acid and lyophilised. The peptide was loaded on a reverse-phase HPLC column and eluted with a gradient from 0 to 60% acetonitrile as shown in the inset.

Table 1

Binding of iodinated peptide to anti-nAChR sera

NRS	cpm bound					
	Antisera to native nAChR				Antisera to denatured nAChR	
	TB7	TB8	TB20	TB22	TB17	TB18
1056 ± 289	1039 ± 268	1013 ± 269	1165 ± 99	1062 ± 132	5979 ± 427	9592 ± 804

Assays were performed as described in section 2. Results are the mean ± SD of 6 determinations

Table 2

Binding of different antigens to anti-peptide sera

Rabbit	cpm bound			
	Peptide	nAChR	SDS-denatured nAChR	Acid-denatured nAChR
TB31	54261	1000	537	5658
TB32	106578	10383	8285	17115
TB33	7915	483	460	2452

The assays were performed as described in section 2. The antisera are from rabbits immunised with 100 μg peptide-KLH (TB31), 770 μg peptide-KLH (TB32) or 100 μg peptide-RSA (TB33). All serum samples are from blood taken 7 days after the third injection. Background counts using NRS have been subtracted

Table 3

Binding of anti-peptide serum to denatured nAChR

Iodinated antigen	cpm bound		
	TB32	TB7	NRS
(a) Receptor	5502 ± 488	58082 ± 1390	1298 ± 123
Receptor + toxin	6353 ± 372	97950 ± 4232	2292 ± 324
(b) Non-absorbed nAChR	17883 ± 1998	154127 ± 6675	3815 ± 142
Toxin-absorbed nAChR	35890 ± 2972	96172 ± 4526	8577 ± 14

The assays were performed as described in section 2. Complexes of radiolabelled nAChR and radiolabelled toxin were formed as described. Absorption of the nAChR was performed by incubating labelled nAChR (500 μl, 65 pmol) with α-cobratoxin beads (500 μl, 30 nmol toxin) for 2 h at 22°C. The results are the mean ± SD of 3 determinations

denatured receptor. Competition experiments between unlabelled peptide and iodinated receptor for a limited amount of anti-nAChR antibodies confirmed this result, and also showed that as much as 33% of antibodies to denatured receptor recognise this peptide (0% block of TB7, TB8, 25% of TB17, 33% of TB18). The iodinated peptide also did not bind to any of 3 monoclonal antibodies directed to the MIR.

In the converse experiment, antibodies raised against the peptide were found to react with peptide and denatured receptor (table 2). No binding

was observed with antisera to carrier protein alone (not shown). Binding was also observed with receptor which had not been deliberately denatured; this however was found to occur only with receptor which did not bind iodinated toxin and could not bind to an α-cobra toxin resin (table 3), nor was binding inhibited by incubation of the receptor with unlabelled α-bungarotoxin (12817 + 264 cpm bound without toxin present, 11893 + 328 cpm in the presence of a 1000-fold molar excess of toxin), showing that this reaction was exclusively with denatured receptor in the nAChR preparation.

4. DISCUSSION

The peptide sequence 161–166 of the α-chain of the nAChR has been proposed as a major antigenic site [5,8,9]. We here show that a synthetic peptide consisting of residues 151–169 (this sequence being identical in *T. californica* and *T. marmorata*) does not bind to antibodies to the nAChR, clearly demonstrating that this site is not an important antigenic site on the molecule. Similarly, no binding was observed to any of 3 monoclonal antibodies to the MIR. These 3 antibodies each bind to slightly different regions of the MIR. Two are known to bind to the α-chain and all react with mammalian nAChRs [3]. It is interesting to note, however, that this peptide does represent a major antigenic site on the denatured molecule. The postulate that this peptide might be important antigenically stems only from the hydrophilicity profile of the molecule. Authors in [24] suggested that the major hydrophilic peak of a molecule is invariably an antigenic site (not necessarily a major antigenic site). Their work was restricted to relatively small soluble proteins, whereas the receptor is a large multichain transmembrane protein. Sequencing of the α-chain genes of chicken [25] and human [9] nAChR shows that the sequence corresponding to peptide 161–166 is interrupted by an intron. Sequences coded by different exons have been suggested to function as different domains of the overall structure [26]. If this were the case, the peptide in question might easily be located in a cleft between two domains, explaining why anti-peptide antibodies do not bind to the native receptor. The alternative explanation for this would be that the anti-peptide antibodies simply do not have the correct conformation to bind to native receptor. However, production of monoclonal antibodies to a number of peptides has demonstrated that as many as 63% of anti-peptide antibodies can bind to the native protein [27]. We ourselves, using polyclonal antisera to another receptor peptide, find between 20 and 90% reactivity with intact receptor (in preparation). The use of hydrophilic peaks as a means of choosing candidates for antigenic sites in complex proteins must therefore be treated with caution. Comparison of the DNA sequence of the genes encoding the chicken [25] and human [9] α-chain and chicken γ-chain (M. Ballivet, personal communication) with the corresponding hydrophilicity profile for the very similar sequence from *T. californica* shows an invariable correspondence between the presence of an intron in the DNA and hydrophilic peak in the polypeptide sequence. It is conceivable therefore that the majority of hydrophilic peaks in such proteins, rather than representing antigenic sites, reflect a more open, hydrophilic region between different domains of the protein.

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